CHAPTER 14

Non-Lethal Methodology for Detection of Fish Pathogens

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I. Introduction

Fish health plays a key role in monitoring, evaluating and protecting the health of all aquatic animals within an ecosystem whether it relates to restoring depleted populations or the recovery of threatened and endangered (T&E) species. For this reason, fish species involved in special recovery projects and those of special management concern (T&E) have been targeted as high priority for sampling criteria outlined in the National Wild Fish Health Survey (Survey).

A. The Endangered Species Act of 1973 was enacted to protect and enhance the recovery of endangered (in danger of becoming extinct) and threatened (likely to become endangered) species. The Act prohibits harmful actions to any endangered or threatened plant or animal species. Increasing numbers of fish species are being listed under the Endangered Species Act (ESA). Biologists participating in the Survey will be responsible for the proper treatment of T&E species that are captured for sampling and other reasons.

B. Permitting - The Secretary of the Interior, through the Regional Directors of the U.S. Fish and Wildlife Service, may issue permits for the taking and possession of T&E species, under certain circumstances.

Fish Health work, whether lethal or non-lethal to the fish collected, may be considered a harmful action which is governed by this permitting process. All personnel involved in collection of samples for the Survey should contact their regional ESA permitting office or recovery team to determine how the Act applies to the work they will be performing, should T&E species be encountered. Decisions on lethal or invasive sampling techniques discussed herein should be made with the participation of the collecting field biologists and the specific recovery team involved.

For more detail, see 50 CFR 17, Code of Regulations, 50 CFR 17.22-17.32 and the Endangered Species Act.

C. Validity and Sensitivity - Many concerns must be considered when evaluating results from non-lethal versus lethal fish health sampling protocols:

1. Sensitivity - in many cases detecting of pathogens from blood serum is less sensitive than internal tissues. In other cases, detection of an organism may be enhanced by utilization of mucus or serum.

2. Validity - specific protocols for detection of fish pathogens using non-lethal sampling means are scarce, and many of those that are utilized have not been validated. Clearly, more research must be accomplished to improve sensitivity and validity of non-lethal detection protocols available in the literature.

3. Statistical concerns - most of the stocks being considered for non-lethal fish health sampling are valuable and/or few in number, further reducing the sensitivity for

detection of any particular pathogen which may be carried by members of that population.

II. Non-lethal Assay Methods to Employ

A. Most of the tissues and materials listed in the table in Section E below can be processed and analyzed in the laboratory according to the procedures described within the chapters referenced in the chart. Several procedures are not described in detail within this manual. An attempt will be made, therefore, to detail them within this chapter of the manual.

B. Considerations for virology:

1. Cell Lines: An impressive number of fish cell lines have been and continue to be developed and established by professionals in the fish health community. Many of these lines are catalogued in the American Type Culture Collection (ATCC). The ATCC database can be queried on the Internet for availability and purchasing information at http://www.atcc.org.

   Additional cell lines, which may not be available at ATCC, are reviewed by Fryer and Lannan (1994).

2. NOTE: the detection of virus from water samples using an adsorption-elusion technique (as listed in the chart) is extremely labor intensive, and therefore only the literary reference for this procedure is given (McAllister & Bebak, 1997).

C. Considerations for Bacteriology:

1. Selective culture Media - a brief list of several media, which are considered selective for culturable bacterial fish pathogens, is given in Section III of this chapter.

2. Part III and IV of this Chapter describe, in detail, procedures for detecting bacteria from fish mucus and water samples.

D. Considerations for parasitology - any material collected non-lethally can be examined microscopically for parasites. Tests using PCR can be conducted to detect Survey target parasites such as *Myxobolus cerebralis* and *Ceratomyxa shasta*. See manual chapters referenced in the chart below (Section E).

E. General Non-lethal Sampling Considerations - Below is a chart which lists seven main forms of fish tissue and other material that can be collected non-lethally and examined for fish pathogens targeted for the Survey. The protocols chapters are referenced.

Sample Material	Target Pathogens	Protocols	Chapter Reference
Coelomic Fluid	Viral pathogens	Cell culture/ PCR	Chapter 10 & 11
	R.salmoninarum	ELISA/ PCR	Chapter 6 & 7
	Bacterial Pathogens	BHIA culture/ serology	Chapter 5
Blood and Blood Serum	Viral pathogens	Cell culture/ PCR	Chapter 10 & 11
	R. salmoninarum	ELISA/ PCR	Chapter 6 & 7
	Bacterial pathogens	BHIA culture/ serology	Chapter 5
	Parasites	Microscopy/PCR	Chapter 8
Fecal /Intestinal Lavage	IPN	Cell culture/PCR	Chapter 10 & 11
	R. salmoninarum	KDM culture/PCR	Chapter 6 & 7
	Bacterial Pathogens	BHIA culture/ serology	Chapter 5
	Parasites (*C. shasta*)	Microscopy/PCR	Chapter 8
Mucus*	IHN, IPN	Cell Culture/PCR	Chapter 10 & 11
	Bacterial Pathogens	BHIA culture/ serology	Chapter 5
	Parasites	Microscopy	Chapter 8
External Lesions	Viral pathogens	Cell Culture/PCR	Chapter 10 & 11
	Bacterial Pathogens	BHIA culture/ serology	Chapter 5
	Parasites	Microscopy	Chapter 8
Water/Sediments**	IPN* (see references)	Adsorption-elusion	
	Bacterial Pathogens*	Filtration/culture/ serology	Chapter 5
	M. cerebralis	Microscopy/PCR	Chapter 8
Tissue Biopsy: Gill*, fin, opercula	Parasites (Including *M.cerebralis*)	Microscopy/PCR	Chapter 8

* with special considerations explained within this Chapter.
** although detection does not come directly from fish, examination of water and sediments can indicate the presence of a pathogen in a particular watershed.

III. Non-lethal Detection of Bacterial Pathogens in Mucus of Fish

The bacterial fish pathogen *Aeromonas salmonicida* can be readily isolated from the mucus of salmonids using the following techniques. Some success at isolating *Yersinia ruckeri* and *Flavobacterium columnare* has also been observed by field personnel. Bacteria can be detected using simple swab/streaks onto agar media, or they can be quantified through serial dilution in PBS. Quantification can help reflect the level of systemic infection in some fish. The following methodologies have been adapted from Cipriano et al.(1992).

Non-Quantitative Method:

1. Mucus is scraped gently from the lateral surface of the fish with a 10Fl inoculating loop, and streaked directly onto the agar media of choice, according to the target pathogen(s) for detection.

2. Alternatively, commercially prepared transport swabs (see source list) can be used to collect mucus specimen, for later streaking onto agar media. Follow manufacturer's instructions on use of the swabs. Be sure to store transport swab samples cold and streak within 24 hours of specimen collection.

Dilution Plate Counts from Non-Lethal Mucus Samples from Fish:

Small samples of mucus can be weighed and diluted in PBS, then plated on an appropriate agar medium. After incubation, colonies of target bacterial pathogens can be quantified and reported as colony forming units per gram of mucus (cfu/gm).

1. With a sterile scalpel or bladed instrument, gently collect a small amount of mucus from the lateral surface of the fish.
   a. Place into a pre-weighed sterile culture tube and keep sample cold until processing can be accomplished.
   b. Determine the weight of the sample by subtracting the tube weight from the gross weight after sample is collected.

2. Make a 1:10 dilution according to tissue weight with phosphate buffered saline(PBS). Homogenize tissue with rigorous pipetting motions.

3. Make serial $\log_{10}$ dilutions of the 1:10 dilution in PBS: Fill micro titer plates with 90Fl per well PBS (4 wells per sample will be needed), or any other vessel with appropriate volume to accomplish 10 fold dilutions.

4. Add 10Fl of the 1:10 dilution tube to the first micro titer well

5. Make 10 fold dilutions by taking 10ul from the first well and placing it in the second.
   a. Change pipet tips in between dilutions.
   b. Do the same from the second to third to fourth wells or tubes of PBS.

6. Prepare media plates with appropriate labels. It is helpful to space the numbers 1 through 5 around the edge of the plate and in the middle to aid in placement and tracking of each dilution.

7. Plate all dilutions, using the same tip but working from the fourth dilution backwards to the higher dilutions. 10Fl drops are most readily absorbed by the agar, but larger volumes can also be plated and spread onto individual media plates.

8. Keep plates upright until the fluid of every drop has been absorbed. Then turn them over and incubate appropriately according to optimal conditions required by the target pathogen.

9. Quantify and isolate bacterial colonies produced from samples:
   a. Drops will vary in bacterial load depending upon the sample.
   b. Pick a spot that contains a countable number of colonies.
   c. Pick representative isolates of each colony type observed for characterization and identification.
   d. Count each colony type and record on media tubes as dilution number times number of colonies counted (example: 1 X 16).

10. Calculate colony forming units per gram mucus (cfu/gm):
    a. cfu/gm = colonies counted X dilution factor
    b. example: 30 colonies counted in 10^{-2} dilution =
       30 X 10^{2} per 0.01 gm (10^{-2} contains 0.01gm of mucus)
    or 30 X 10^{4} per 1.00 gm (decimal moved)
       3 X 10^{5} cfu divided by original sample weight = cfu/gm tissue

Quantification of bacteria on the surface of fish can give a good indication of the level of internal infection that may exist. For instance, when *A.salmonicida* exceeds 10^{3} cfu/gm mucus on a Salmonid, there is a strong possibility that the fish has a systemic infection that could be lethal to the fish. This work has been done under normal fish culture circumstances, however, and adjustments to determine lethal versus carrier infections in wild fish may be necessary.

C. Materials and Sources

BACTI-SWAB™ Modified Stuart's Transport Medium
available from Remel (800-255-6730).

IV. Procedures for the detection of Bacteria in Filtered Water Samples

Water can be examined for a variety of bacterial species, including those that can serve as pathogens to fish. Water can be sampled directly from streams, ideally in areas where more fish are congregated, such as below pools, logs, and other habitat fish use for shelter. Sampling at effluents of fish culture facilities can also provide information on cycles of pathogens shedding into the environment. Bacteria can be enumerated on the media plates and reported as colony forming units (cfu) per milliliter (ml). The following methodologies have been adapted from Ford(1994).

A Methods:

1. Collect water sample in sterile 200 ml container.
   a. Take care not to contaminate sample with hands
   b. Keep cold until filtering and plating can be accomplished

2. Wipe filter unit with alcohol and carefully load with .45um pore filter paper (grid side up). Take care not to contaminate filter by touching with hand - use forceps that have soaked in alcohol.

3. Filter several dilutions of sample with enough sterile distilled water to make a 100 ml total volume.
   a. Start with the most dilute volume of sample. For example: if plating 1,10 and 100 ml of water, fill filter unit with 99mls DH_2O and transfer 1ml sample. The next dilution (10 ml sample in 90 ml DH_2O) can be done without disinfecting filter unit. Do, however, wipe it with alcohol between different water samples.
   b. The number and volume of dilutions should be adjusted with the quality of water samples: murky water will contain a lot of bacteria, and therefore, smallest filtered volume may have to be 0.5 or 0.1 ml
   c. Always dilute with sterile distilled water to bring total filtered volume to 100 ml for consistency and even distribution of bacteria.

4. After filtering, plate samples onto desired media:
   a. With disinfected forceps, gently grasp each filter paper from unit and place grid side down onto agar media surface, removing any air bubbles with forceps.
   b. The filter need remain for only a few minutes before it can be removed and discarded with clean forceps.

5. Incubate plates for appropriate time and temperature depending on target organisms.

6. Perform bacterial counts and isolations directly from plates. Frequency plots and histograms of the major bacterial genera can be plotted as well as the number of cfu/ml of target pathogen in a particular sample.

Materials and sources:

Sample Containers (Sterile)240 ml (Thomas Scientific/6186-M40)
Nalgene Filter Apparatus (Thomas Sci./4618-N60)
Nalgene Filter Apparatus (Thomas Sci/ 4618-N62)
Filters (Sterile), 47mm, 0.45Fm pore (Thomas Sci./4626-J20)
Filter Pump (aspirator type - VWR/ 28610-008) or a vacuum pump
1 ml Pipets (Sterile-Fisher Sci./13-678-11A)
Coomassie Brilliant Blue (R250-Sigma/B-0149)

Other equipment needed: Forcep
Alcohol (70% isopropyl)
Sterile Distilled Water
Agar Plates (depending on target pathogens)

C. Media Suggestions:
The following are media and components which select for, or enhance isolation of the given target pathogen. A literary reference is provided for each.

1. ***Aeromonas salmonicida*:**

Tryptic soy agar (TSA)-commercial media - follow preparation instructions.

Coomassie Brilliant Blue agar (CBBA) - (Cipriano & Bertolini, 1988).

TSA	40 g
Coomassie brilliant blue R-250(CBB)	0.1 g
dH_2O	1.0 L

Autoclave for 15 min at 15 psi (121°C) and pour into petri plates. *A. salmonicida* colonies will appear as dark blue, friable colonies after 48 hours at 20°C.

2. ***Flavobacterium psychrophilum, F. columnare:***

Tryptone Yeast Extract Supplemented (TYES) - (Holt & Amandi, 1989)

Tryptone	4.0 g
Yeast Extract	0.4 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2 \cdot 2H_2O$	0.5 g
Agar	10.0 g
dH_2O	1.0 L

Dissolve ingredients and adjust pH to 7.2. Heat to boiling for 1 minute. Autoclave for 15 min at 15 psi (121°C) and pour into petri plates.

Tryptone Yeast Gelatin (TYG) - (Bullock, et al, 1986)

Tryptone	2.0 g
Yeast Extract	0.5 g
Gelatin	3.0 g
Agar	15.0 g
dH_2O	1.0 L

Dissolve ingredients and adjust pH to 7.0. Heat to boiling for 1 minute. Autoclave for 15 min at 15 psi (121°C) and pour into petri plates.

3. ***Yersinia ruckeri:***

TSA, BHIA - both commercially prepared

Shotts-Waltman (SW) -(Waltman&Shotts, 1984)

Sodium Chloride	5.0 g
Tryptone	2.0 g
Yeast Extract	2.0 g
Tween 80	10 ml
$CaCl_2$ @$2H_2O$	0.1 g
Bromthymol Blue	0.003 g
dH_2O	950 ml
pH to 7.4 and add:	
Agar	15 g

Heat to boiling. Autoclave for 15 min at 15 psi (121°C).Add 10 ml of 0.5g/ml sucrose solution which has been filter sterilized. Refrigerate poured plates until use.

Y. ruckeri will produce a green colony with a zone of hydrolysis (precipitation of calcium oleate from Tween 80). Always confirm colony identity with biochemical characterization of isolates. (Type II will not hydrolyze Tween 80).

4. ***Renibacterium salmoninarum:***

Kidney Disease Medium (KDM2) - (Evelyn, 1977)

Peptone	10.0 g
Yeast Extract	0.5 g
L-Cysteine HCl	1.0 g
Distilled water	1000 ml
Adjust pH to 6.5	
Agar	15.0 g

Autoclave for 15 minutes at 121°C. Cool to ~ 50°C and add:

FBS	200.0 ml

The following volumes of antibiotics can also be added to the KDM2 (SKDM) to reduce overgrowth from other bacterial organisms (Austin, et al. 1983).

Cyclohexamide	4.0 ml (see below)
D-cycloserine	1.0 ml
Polymyxin B-sulfate	2.0 ml
Oxolinic Acid	1.0 ml

Prepare the above antibiotics following these formulas:

Antibiotic Solution Preparation:

	grams	ml
Cyclohexamide	1.2	96 dH_2O
D-Cycloserine	0.3	24 dH_2O
Polymyxin B-sulfate	0.3	24 dH_2O
Oxolinic Acid	0.06	24(5% NaOH)

Researchers found that variable lots of peptone could adversely effect the ability to successfully culture *R.salmoninarum* using these media. Evelyn et al. (1990) reported on the use of a metabolite solution from KDM broth containing *R.salmoninarum* (autoclaved or filter sterilized using 2 Fm pore size) and added to the KDM media at 2% (v/v). This "metabolite" has shown to improve success in culturing this organism, and seems to negate the adverse effects of poor peptone lots used in the media.

5. *Edwardsiella ictaluri*

S-W *E. ictaluri* Selective Media - (Shotts & Waltman, 1990)

Tryptone	10 g
Yeast Extract	10 g
Phenylalanine	1.25 g
Ferric ammonium chloride	1.2 g
Bromthymol blue	0.003 g
Bile salts	1.0 g
Agar	15.0 g
Distilled water	980 ml

Dissolve ingredients by boiling, then cool to 50°C and adjust pH to 7.0. Autoclave for 15 min at 15 psi (121°C).

Cool to 50°C again and add mannitol (filter sterilized) to 0.35% (v/v) and colistin sulphate to 10 Fg/ml.

Interpretation:
Proteus species will produce brown colonies (caused by phenylalanine and ferric ammonium chloride).
Serratia and *Aeromonas* will ferment mannitol producing yellow colonies.
Edwardsiella ictaluri will produce translucent, colorless colonies.

V. Method for Non-Lethal Gill Biopsy

Gill filaments can be removed from fish while under anesthesia with little injury to the fish. The tissue can be examined directly under microscopy for parasites, preserved for histology, or frozen for examination using other diagnostic methods, such as PCR for *Myxobolus cerebralis*. The following protocol has been adapted from methods used for collection of gill Na^+, K^+-ATPase activity measurements in salmonids (McCormick, Personal communication).

A. Methods

1. While fish is under anesthesia, place on a moistened chamois cloth to minimize scale loss and damage. A right handed individual should place the fish on its right side so that the head is to the left and tail to the right.

2. Gently pull back the operculum with rounded forceps. A cartilaginous septum (present in some species such as salmonids, but not in others) holds filaments together for one-half their length.

3. Using a fine pointed scissors, remove 4-6 filaments just above the septum from a fish weighing between 20 and 80 grams (remove more or less tissue for larger and smaller fish, respectively).
   a. With the operculum reflected, isolate several filaments with the open blades of the scissors (see Figure 1).
   b. Turn scissors so that they are perpendicular to the filaments and cut in a single quick motion.
   c. To retain the filaments on the scissor blades, turn the scissors slightly as you finish cutting.

   Take care not to crush sample or remaining filaments. if there is any movement from the fish, be sure to retract forceps and scissors quickly to avoid injuries. Return fish to fresh water immediately.

4. Transfer filament samples to appropriate containers for storage until assays cane be performed.

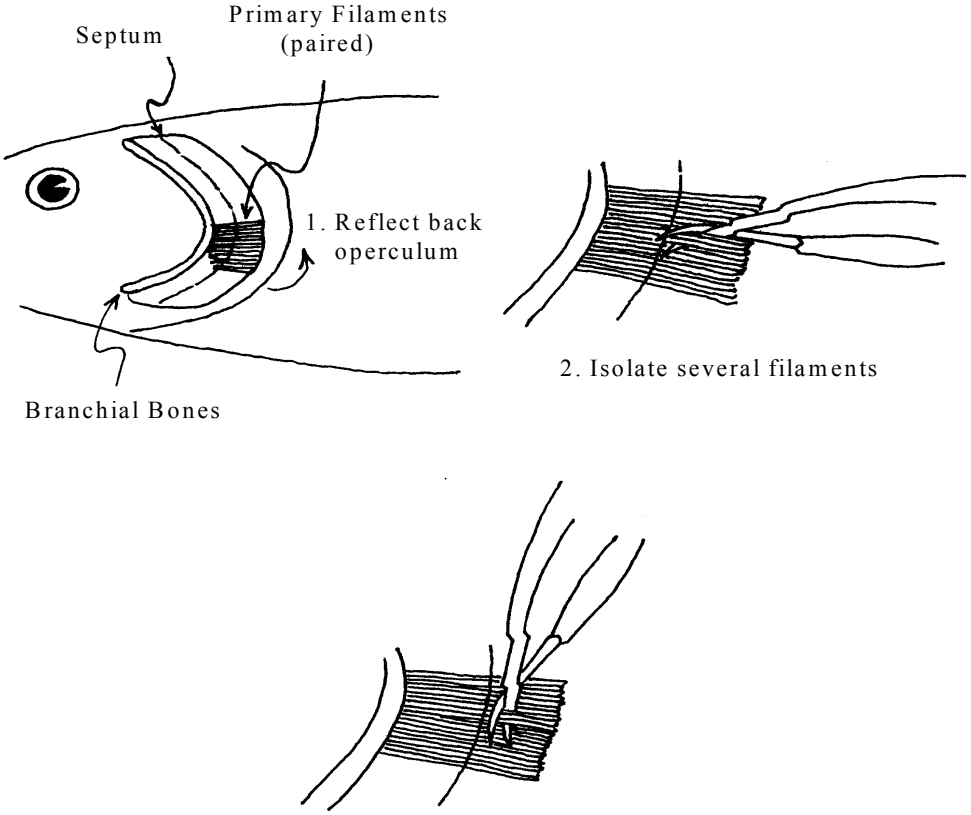
B. Recovery of fish subject to gill biopsy: Upon return to the water, the fish may bleed slightly for up to one minute. Excessive bleeding, beyond one minute is usually associated

with cutting to deeply into the filaments (i.e. below the septum). Even excessive bleeding does not usually result in mortality.

3. Materials

Chamois Cloth
Rounded forceps
Fine point scissors
(Vannas eye scissors - 7mm curved blade, Sicoa-phone 201-941-6500, Cat.# OM-1401)
Collection vials
Fish anesthetic

Figure 1 – Non-Lethal Gill Biopsy



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